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The Effects of Apoptosis and the Cell Cycle Arresting of Valproate and Nicotinamide on U87 Cell Line

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ABSTRACT

Epigenetic changes such as histone acetylation changes affecting genes play an important role in the development of various human cancers. HDAC inhibitors are now approved by the FDA for the treatment of cancer malignancies as well as clinical trials for tumors. Histone deacetylases play a role in the onset and progression of many cancers through effects on cell cycle, epithelial differentiation and apoptosis. We examined the antiproliferative effects of valproate with a combination of nicotinamide in human glioblastoma U87 cell line. The MTT assay showed that valproate at 0.5 mM, when used alone weakly, suppressed proliferation of cells (39% ± 3.05) and combination treatment of valproate + nicotinamide strongly suppressed cell proliferation (60% ± 3.5). Flow cytometric analysis showed that in the treatment of cells when the combination of valproate and nicotinamide was used, it showed more inhibitory effects on cell viability than when valproate alone was used. Also, western blot analyses have done to study the acetyl-histone H3 levels, and quantitative Real time PCR were performed on expression of p21 gene in U87 cell line. The combination treatment of valproate + nicotinamide enhanced the expression of p21 gene. The biological response of the cell line correlated with the increase of histone H3 acetylation after nicotinamide and valproate application. The findings indicate that co-administration of valproate and nicotinamide can have inhibitory effects on the growth and proliferation of human glioblastoma U-87 cells and may be a suitable option for new treatments for brain tumors.

Keywords: U87 cell, HDAC, HAT, Valproate, Nicotinamide

INTRODUCTION

Today, close to fifteen HDAC inhibitors are being studied in early studies for clinical use in cancer treatment [1]. The science of using histone deacetylase to treat cancer is a very novel science. In some cases, the use of histone deacetylase inhibitors are a very bright result in stopping tumor growth, yet the exact mechanism is unclear [2]. The use of histone deacetylase inhibitors is important in several aspects: 1) it opens up the chromatin-packed structure and facilitates access to DNA-damaging materials, resulting in cell death. 2) Stop the cell cycle at the G1/S stage. 3) Induces apoptotic mechanisms. 4) Inhibit angiogenesis, and 5) have a direct effect on the activation and inactivation of tumor suppressor gene and oncogenes [3,4].

Histone deacetylase contains an old family of enzymes that play an important role in many biological activities. These enzymes interfere with the process of angiogenesis, metastasis, and apoptosis [5]. They are overexpressed in many types of tumors. HDACs remove acetylate group of ε-amino-lysines located in the N-terminal tail of the histones. In addition to chromatin density, HDACs regulate the function of different proteins [5]. The HDAC family consists of 18 HDACs that are divided into four groups: Class I, including HDAC 1, 2, 3 and 8; Class II HDAC includes 4, 5, 6, 7, 9 and 10; HDAC Class III includes sirtuin (1-7); and Class IV contains HDAC 11 (class I, II and IV). In mammals, the seven homologs Sir2 (Sirtuin 1-7 or SIRT1-7) are known to be primarily HDAC (SIRT1, SIRT2, SIRT3, SIRT5), or monoribosyltransferase activity (SIRT4 and SIRT6) that contain histone and non-histone proteins in different stages of cellular staining. SIRT1 mammals have a widespread function in cells [6]. SIRTs play an important role in modulating the activity of tumor suppressor genes. For this reason, these molecules are the

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appropriate therapeutic targets for the development of anticancer drugs. However, despite the development of several effective SIRT inhibitors, certain mechanisms of cellular activity and targets for these proteins are not known [7].

The HDAC inhibitors represent a class of targeted molecules that can modulate the epigenetic changes in histone proteins and thus affect the expression of the gene [8]. HDAC inhibitors can be classified into several groups, including short chain fatty acids, small hydroxylate molecules, cyclic peptides, benzamide, titanium compounds, ketones and other hybrid compounds. Some HDAC inhibitors are limited in terms of therapeutic side effects due to toxic side effects in high doses. HDAC inhibitors can cause the death of adenocarcinoma cells [9]. Other researchers have reported that histone deacetylase are potentially attractive molecular targets for the sensitivity of cancer cells to radiation therapy. These inhibitors can affect gene expression patterns, differentiation, apoptosis of cancer cells, and their sensitivity to therapeutic agents [10]. Studies have recently shown that HDAC Class I and II inhibitors, such as SAHA, can result in apoptosis of tumor cells in a variety of cancers of the lung, breast cancer, pancreatic cancer, and liver through apoptosis. It has been shown that combination therapy with a low amount of SAHA can increase the sensitivity of the cell to 5-fluorouracil, especially in chemotherapy-resistant cancers [11].

Valproate is a voltage dependent sodium channel blocker, which increases the amount of gamma amino butyric acid in the brain; although it's exact mechanism is unknown. One of the most common side effects is digestive disturbances such as indigestion, nausea, skin rashes and anemia [12]. Valproic acid and its salt derivative, sodium valproic acid, have less side effects than other histone deacetylase inhibitors. Twenty four years ago, valproic acid was introduced as an anti-epileptic drug in the United States. Valproic acid, or 2-propylptanoic acid, is a member of the family of histone deacetylase inhibitors [13]. These inhibitors are classified as a family of aliphatic acids. In addition to valproic acid, phenyl butyrate and sodium butyrate are in this group. Sodium butyrate inhibits the cell cycle in different types of tumor cells and can induce apoptosis in them. In 1882, valproic acid was synthesized for the first time, but by 1967, scientists had noticed its therapeutic effects on patients with epilepsy and seizure [14]. Valproic Acid was first approved in 1982 for use in cancer treatment. In 2001, two distinct research groups led by Phiel and Gottlicher provided reports on the histone deacetylase inhibitory properties. Valproic acid is used to treat various types of cancers either alone or in combination with other drugs. In 80% of patients with breast, lung, and ovarian cancer, the combination of two compounds of valproate and all *trans* retinoic acid is used for treatment. Sodium butyrate, phenyl butyrate and sodium valproate have been shown to increase the sensitivity of cancer cells to radiation therapy [15].

Valproate can cause hyperacetylation of H3 and H4 histone N-terminals in vitro and *in vivo* and inhibit HDAC activity at 0.5 mM concentration. Valproate shows strong antitumor effects in a variety of in vitro and in vivo systems, by modulating multiple pathways, including cell cycle stop, apoptosis, angiogenesis, metastasis, differentiation, and aging [16]. The effect of valproate on acute myeloid leukemia cells and chronic myeloid leukemia cells and acute lymphoblastic leukemia cells has been studied. The precise molecular activity of valproate in the treatment of leukemia has not yet been justified [17]. Nicotinamide, a form of vitamin B3 amide (niacin), has long been associated with neurodevelopment, survival, and central nervous system (CNS) function, which has been implicated in neural death and neuroprotection [18]. Nicotinamide is an effective and safe agent for lung cancer dietary prevention at both early and late stage carcinogenesis. Combination chemoprevention with these agents is a well-tolerated and effective strategy which could be clinically advanced to human studies [19]. In this study, we tried to determine the role of this compound in the proliferation and apoptosis of U87 cells. The combination therapy of valproate with nicotinamide may improve efficacy while reducing side effects. The purpose of the present study is to develop a new strategy to treat brain tumor.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Human glioblastoma cell line U87 was purchased from Pasteur Institute (Iran). U87 was cultured in DMEM medium (Gibco; 10829-018) supplemented with 10% fetal bovine serum (Gibco; 10439-024) and 100 µg ml^{-1}

streptomycine-peneciline (Gibco; 15070-0663) at 37 °C in a humidified atmosphere of 5% CO_2.

Cell Proliferation Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma; M5655) assay was performed to evaluate cell growth. 10^5 cell/ml was seeded into a 96-well plate 48 hours prior to experiment at use. After 48 hours cells were treated with nicotinamide (Sigma; N0636) and valproate (Sigma; P4543) at different concentrations for 48 h. After treatment, with valproate and nicotinamide, MTT (0.5 mg ml^{-1}) dye solution was added into the 96 well plate. The cells were then incubated for 3 hours at 37 °C. At the end of this incubation, 100 µl of SDS was added. The absorbance at 540 nm was determined for each well using a microplate ELISA reader. Each experiment was conducted in triplicate. The IC_{50} values defined as the drug concentration that inhibits 50% of growth compared to untreated cells, were then determined for each HDACI using the dose-dependent curves [20].

Real-time PCR

Total RNA was extracted using TRIzol (Sigma-Aldrich) according to the manufacturer's instructions. Synthesis of cDNA was carried out with M-MuLV reverse transcriptase and oligo (dT) primers (Fermentas, Burlington, Canada). Reaction mixtures for PCR included 200 µM dNTPs, 2.5 µl cDNA (5 µM), 1 × PCR buffer (AMSTM, Cinnagen, Iran), and 0.5 µM of both forward and reverse primers and 1 U Taq DNA polymerase (Fermentas, Maryland, MD). PCR amplification was performed using a standard procedure. Amplified DNA fragments were electrophoresed on 2% agarose gels, stained with ethidium bromide and illustrated on a UV transilluminator (UVIdoc, Cambridge, UK). Real-time PCR was performed using a standard SYBR Green PCR kit (Fermentas) protocol on a RotorGene 6000 instrument (Corbett, Sydney, Australia). Data was normalized to an endogenous control gene (HPRT) and calibrated to 12-d cultured cells. All reactions were run in triplicate and the threshold cycle average was used for data analysis by Rotor-gene Q software (Corbett). The relative mRNA expression levels were calculated based on the delta CT method. The sequences of the primers used for the analysis of p21 were as follow: CDKN1A (p21)-fw 5' CCA GCA TGA CAG ATT TCT ACC 3'; CDKN1A (p21)-rev 5' AGA CAC ACA AAC TGA GAC TAA GG 3'; HPRT-fw 5' CCT GGC GTC GTG ATT AGT G 3'; HPRT-rev 5' TCA GTC CTG TCC ATA ATT AGT CC 3'.

Western Blot

Alteration of histone acetylation in presence and absence of the valproate and nicotinamide was investigated by Western blot as described previously [21]. U87 cells were washed with cold PBS once and resuspended in radioimmunoprecipitation lysis buffer (RIPA) containing protease inhibitors cocktail (Roche Diagnostics; Tokyo, Japan). The cell lysate was left on ice for 30 minutes and centrifuged at 14000 rpm for 15 min. The supernatant was obtained and the protein concentration of cells was quantified using the BCA protein assay kit (Pierce). Samples were diluted in loading buffer (10%v/v glycerol; 0.05 M Tris pH 6.8; 2% Sodium dodecyl sulfate (SDS); 0.01% w/v bromophenol blue and 2.5%v/v mercaptoethanol) by heating at 100 °C for 10 min. Equal protein quantities were loaded in each lane in a 12% SDS polyacrylamide gel and electrophoresed. Then, the proteins were transferred to a 0.45 µm PVDF membrane (Millipore, Bedford MA) using a Trans-Blot transfer tank. The membranes were treated with 5% skim milk in a TBST buffer, pH 7.4 (10 mM Tris-HCL, 150 mM NaCl, 0.1% Tween)for 2hr to block non-specific binding and incubated at 4 °C overnight with anti-acetyl-histone H3 rabbit polyclonal antibody diluted 500-fold in TBST buffer or beta-actin (Abcam, mouse Ig G 1:5000), as a control for a protein loading. The membranes were then washed three times, each for 5 min with PBS-Tween (0.1%). Afterward, membranes were incubated with the appropriate horse-radish peroxidase-conjugated secondary antibody (1/2000; Ray Biotech) for 2 h at room temperature followed by three times of washing, each for 10 min. Membranes were developed with chemiluminescence western blot detection reagents (Roch, Indianapolis, IN).

Flowcytometry Assay

We performed cell cycle arrest analysis by Flow cytometry. The cells were evaluated by kit (Roche). The cells are centrifuged for 8 minutes at 1200 rpm. The volume of 1 ml of 70% cold ethanol was added drop wise to the

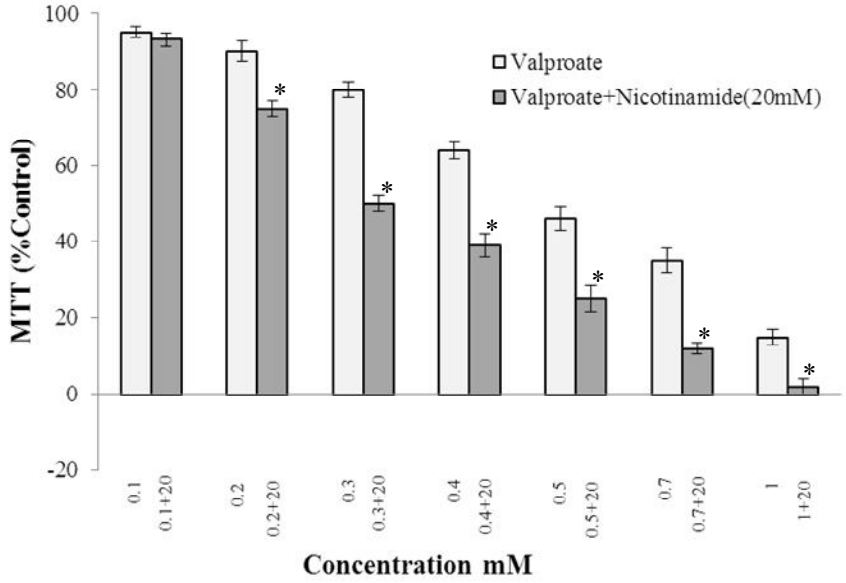

Fig. 1. The effects of valproate and nicotinamide on cell cytotoxicity. The U87 cells were seeded in 96-well plates (10^4 cell/ml) in DMEM culture medium. After 48 h of seeding, cells were exposed to different concentrations of valproate (0.1, 0.2, 0.3, 0.4, 0.5, 0.7 and 1 mM) and nicotinamide (20 mM). After 48 h of exposure, the cytotoxicity was determined using colorimetric MTT assay. Data are shown as mean ± SE of three independent experiments in triplicate format, *Represents the mean difference with $p < 0.001$ in comparison to DMEM.

cells. The cells are quickly transferred to 20 °C for 24 h. The cells can be kept for at least 6 months. After 24 h, the cells are centrifuged and then extracted with ethanol; it is washed twice with cold PBS. After completion of the wash, the pellet is dissolved in 1 ml of PBS buffer containing 0.5 mg of RNase A and 50 µg of PI, and incubated for 3 hours at 37 °C. After centrifugation, the cells are placed in a PBS buffer and analyzed by flow cytometry (BD, Partec).

Statistical Analysis

Comparison of the effects of various treatments was performed using analysis of variance and the Student's *t* test. Differences with a p value of < 0.05 were considered statistically significant. Experiments shown are the means of multiple individual points (± SEM). To determine synergism of drug interaction, median dose effect isobologram analyses were performed according to the methods of T. C. Chou and P. Talalay using the Calcusyn program for Windows (BIOSOFT, Cambridge, UK). To evaluate the synergism between valproate and nicotinamide, a combination index (CI) was calculated using the following formula: CI = sum of specific apoptosis of single agent treatment/specific apoptosis upon combined treatment. The percentage of specific apoptosis was determined using the following formula: specific apoptosis = (drug induced apoptosis-spontaneous apoptosis)/(100-spontaneous apoptosis) × 100%. Cells are treated with agents at a fixed concentration dose. A combination index value of less than 1.00 indicates synergy of interaction between the drugs; a value of 1.00 indicates additivity; a value of >1.00 equates to antagonism of action between the agents.

RESULTS

Effects on Cell Proliferation

Based on MTT results, 0.5 mM Valproate caused 50% inhibition of U87 cell proliferation after 48 hr of treatment (Fig. 1) and treatment of valproate combined with nicotinamide (VPA + NA) greatly decreased cell proliferation (1.78%). The IC50 value of nicotinamide in U87 was 20 mM. We examined the effect of different concentrations of valproate with nicotinamide on the

Table 1. A combination Index (CI) Calculated with Following Formula: CI = Sum of Specific Apoptosis of Single agent Treatment/ Specific Apoptosis upon Combined Treatment. CI Values < 1, =1 and >1 Mean that the Effect is Synergistic, Additive or Infra-additive Effect, Respectively

Valproate (mM)	Nicotinamide (mM)	CI
0.1	20	0.42
0.2	20	0.36
0.3	20	0.32
0.4	20	0.58
0.5	20	0.70
0.7	20	0.73
1	20	0.90

proliferation of U87 using Calcusyn program (Table 1). The synergism effect is stronger if the calculated CI is closer to 0.1. According to Table 1, the calculated CI value for valproate 0.3 mM is close to 0.3, so we used 0.5 mM valproate and 0.3 mM valproate + 20 mM nicotinamide to further study the antiproliferative activity of the valproate and nicotinamide in U87 cells. There was no significant difference in total cell number of U87 in the presence of 0.1 mM (97% ± 1.5) and 0.2 mM (94% ± 2.6) valproate. These results suggest that combination of valproate and nicotinamide are more effective in suppressing the cell viability of U87 cell line than valproate alone and at lower concentrations.

Effects on Cell Cycle

To investigate the effect of sodium valproate and nicotinamide on the U87 cell cycle, the distribution of treated cells in different phases of the cell cycle in treated cells was investigated using PI color and flow cytometry and its results were based on the intensity of fluorescence emitted percent in Fig. 2 is shown. Cells with valproate 0.7 mM, 25 mM Nicotinamide and 0.5 mM sodium valproate and 25 mM nicotinamide were treated for 48 h. 7% of the control group cells, 8% of the nicotinamide treated cells and 14% of the treated cells with sodium valproate in the G2/M phase, while when the cells were treated with nicotinamide and sodium valproate, this percentage was 52% Receipt. In these cells, the inhibitory pattern of G2/M was observed in the cell cycle.

Effects on p21 Gene Expression

Real time PCR analysis of the cells showed that the expression of suppressor gene p21 was increased. U87 cells treated with 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 1 valproate and 20 mM nicotinamide for 48 h, results have shown in Fig 3. Expression of p21 was increased at 0.5 mM valproate and valproate (0.3 mM) + nicotinamide (20 mM) relative to the control (Fig. 3).

Effects on Histone Acetylation

We first examined the protein acetylation level in U87 after treatment with valproate or nicotinamide. The significantly increased protein level of acetylated histone 3 was detected by western blot in both valproate- and nicotinamide-treated cells (Fig. 4). These results demonstrate that combination of valproate and nicotinamide increases histone 3 acetylation more than valproate alone in U87 cancer cells.

Discussion

Cancer refers to a disease in which cells have uncontrolled growth and proliferation, and this abnormal growth and abnormalities result in abnormal mass

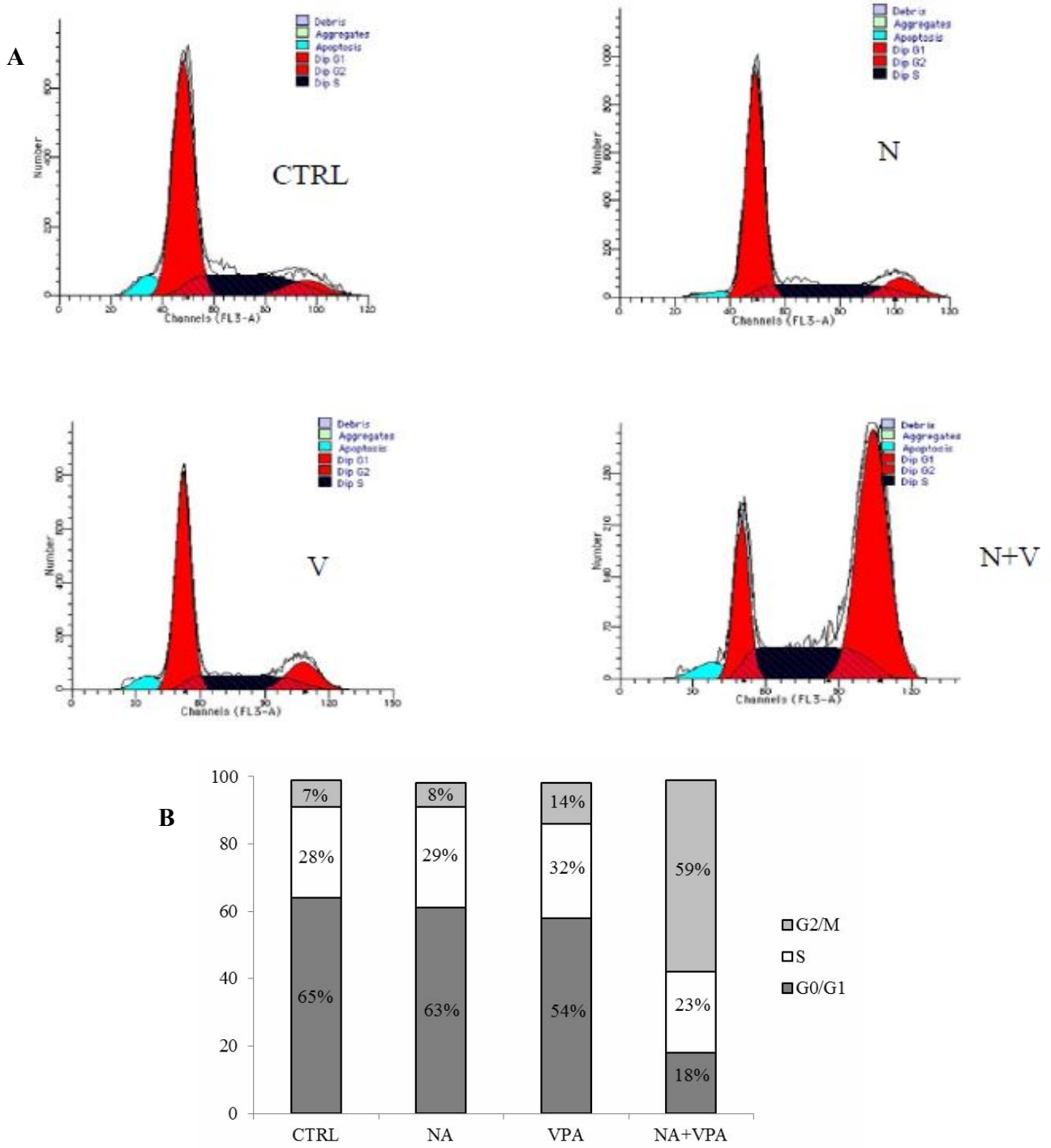

Fig. 2. Effects of nicotinamide and valproate on cell cycle or PI staining. (A) In the DNA histogram, the horizontal axis X (FL3-A) represents the PI fluorescence intensity and the y axis represents the average number of cells in percent representing cellular accumulation at each stage of the cell cycle. (B) In each column, the percentage of different stages of the cell cycle is shown. Control (CTRL), (NA) nicotinamide, (V) valproate, (NA + V) combination simultaneously. The displayed data represents the average of the data obtained from at least 3 separate tests.

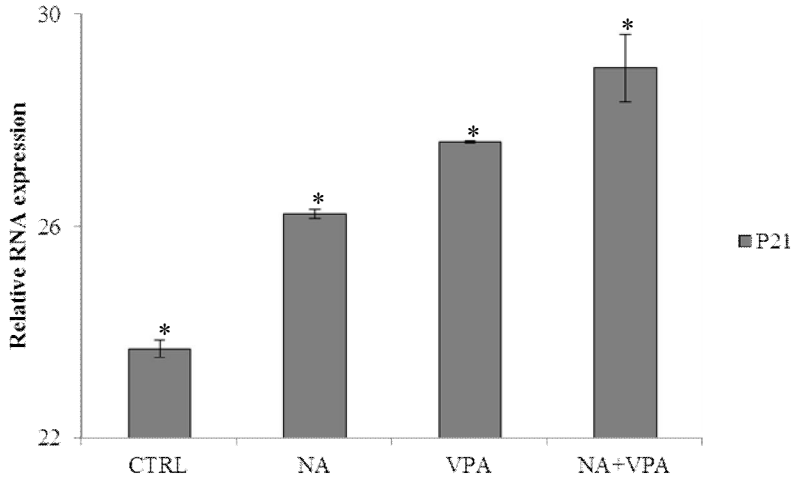

Fig. 3. Effects of valproate and nicotinamide on expression of p21.

Fig. 4. Effect of valproate and nicotinamide on H3 acetylation. U-87 cells were treated with DMEM CTRL), 0.5 mM valproate, 20 mM nicotinamide and nicotinamide (20 mM) + valproate (0.3 mM) for 48 h. The H3acetylated was analyzed by Western blotting.

formation, which is the hallmark of all cancers [22]. Carcinogenesis occurs as a complex process through the activation of carcinogenic genes and the inactivation of tumor suppressor genes. Glioblastoma is the most prolonged and most invasive tumor of the brain [13]. It has a mean neonatal time of about thirteen to 14 months, with only a few survivors over 4 years of age [23]. The U87 cell line is one of the most sophisticated human brain glioblastoma cells. Based on the results of previous studies, brain tumors are the most important type of human tumors. Although these cancers are less prevalent than other cancers, including gastrointestinal cancers, but due to severe complications and lack of proper and high-grade treatment, these tumors are very important [24]. The most common types of brain tumors in the meningeal hyperglycemia are usually benign and astrocytoma likes glioblastoma [25]. According to previous studies, glioblastoma accounts for 15% of the prevalence of brain tumors and is the only way to treat patients by surgery and after chemotherapy [26]. Despite the good progress made in the field of molecular biology of this type of cancer, the treatment of this disease remains unresolved [27]. Researchers now consider histone deacetylase inhibitors as one of the most important anticancer drugs. The first reason for this is that a number of class I and II inhibitors play a role in the proliferation of normal and normal cells. For example, HDAC3 is required for DT40 cell proliferation [28]. Silencing the expression of the HDAC1 gene in mouse embryonic stem cells causes death in the fetal phase and defective reproduction, which is due to an increase in the amount of acetylation and the expression of p21 and p27. In 1999, Valproate was first used as an anticancer drug in clinical studies. The half-life of sodium valproate in plasma is 9 to 18 h [29]. Patients with cervical cancer were the first to use this drug in the clinical phase. In these patients, the H3 and H4 histone acetylation was studied [30]. Hydralazine was also used in combination with valproate. Doctors added to their drug combination with valproate and hydralazine to people with cisplatin treated cervical cancer. Further studies in these patients showed that the side effects of valproate have been

greatly reduced [31]. Valproate increases the cell cycle stop in the G1 stage. In neuroblastoma cells, it has been shown that this drug also reduces the expression of n-myc protein and, on the other hand, affects cell cytoskeleton and prevents cellular and metastatic movements. Until 2001, it was not known that this effect was applied through histone deacetylase. According to the results obtained in this study, the inhibitory effect associated with the simultaneous use of sodium valproate and nicotinamide on glioblastoma cell proliferation had a significant difference with the effects of observation with each of the drugs alone. Decreased metabolic activity of the cells results from a decrease in the number of cells due to cell cycle or cell death. Uncontrolled cell cycle plays a major role in the occurrence of cancer. Cell cycle analysis and confirmatory flow cytometry tests showed that the simultaneous use of valproate and nicotinamide stop glioblastoma cells in the G2/M phase, which is accompanied by an increase in the expression of the negative regulator p21. Real-Time PCR results showed an increase in the expression of p21 after the combined combination of sodium valproate and nicotinamide. Histone deacetylase inhibitors have different functions in the cells. One of the effects of these can be seen in the effect that affects the cycle of the cell cycle. These compounds stop cell proliferation in different cell lines, by holding cells in the G1 or G2/M phase. Histone deacetylase inhibitors cause cyclic-D and cyclin-A to eventually reduce CDK4 and CDK2 activity and stop the cell in the G1 phase. Two other enzyme inhibitors, SAHA and TSA, also increase the expression of p21. It seems that histone deacetylase inhibitors in U87 have the effect of stopping growth through p21. U87 shows a slight increase in the percentage of apoptosis cells added by the addition of valproate, but when nicotinamide is also added to valproate, the percentage of apoptosis cells increases greatly. Treatment of SW-480, CX-1 and WIDR (with clonal cancer origin) with sodium valproate causes the cell cycle to stop in the G2/M phase, which is due to increased expression of p21 [32,33]. Treatment of Bel-7402 (hepatocyte origin) with sodium valproate has shown that low concentration of sodium valproate increases H3 and H4 in the promoter region of p21, which increases the expression of p21 and stopping cells in the G2/M stage [34].

In this study, the expression of p21 in treated cells at the same time as valproate and nicotinamide was 3.7 times, treated with valproate 2.5 times and treated 1.7 times with nicotinamide compared to control group. In U87 cell, because of the inactivation of p53, the expression of p21 increased as a result of an increase in H3 acetyls and independent of p53. The combination of histone deacetylase inhibitors, sodium valproate and nicotinamide inhibits the closure of the chromatin structure in U87 cells, which extends the cells from the G1 phase to the S-phase, and better results in the effects of chemotherapy drugs such as 5-fluorouracil. Vorinostat is one of the other inhibitors of histone deacetylase, which, by increasing the expression of p21, causes the cells to stop in the G1 phase and the apoptosis of the CTCL and HL-60 cells [35]. Nicotinamide acts as a class III histone deacetylase inhibitor, which increases the staining of p53 and its activity. P53 is inactive on the U87, so p21 activity should be increased from a path other than p53. When nicotine amide and valproate are used concurrently, nicotinamide significantly increases the sensitivity of U87 cells to valproate. The effect of valproate is very short, and the presence of nicotinamide helps to better prevent cell proliferation. The amount of H3 acetyls in the U87 cell line has slightly increased after treatment with nicotinamide, which indicates a reduction in the activity of class 3 histone deacetylase, or the sirtuins. When treated with nicotinamide and valproate simultaneously, the amount of H3 acetyls is increased significantly. Valproate in MT-450 and AML cells reduces cell proliferation and reduces metastases [35]. Similarly, in the results of the MTT section, the combination of nicotinamide and valproate reduces the proliferation of U87 cells compared to when nicotinic amide or valproate is used. The effective concentration on inhibiting the proliferation of cancer cells was approximately twice the effective concentration on inhibition of normal cell proliferation. Increasing the expression of p21 is very effective in increasing the sensitivity of cells to anticancer drugs. The study of synergistic effects of histone deacetylase inhibitors, especially those currently used in the clinical phase will reduce the time and cost of developing new drugs and their effects in vitro and *in vivo*. As well as the effects of many side effects on patients.

It can be said that histone deacetylase inhibitors are one of the researchers' hopes for cancer treatment. However, the

efficiency of their use should be carefully checked. Their unique function is to regulate the expression of genes associated with cell apoptosis and cell proliferation, and may be used in combination with chemotherapy in the future. To date, several clinical studies have been conducted in order to use it alone or to combine it with other drugs.

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